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# Functional analysis of lymphocytes subpopulations in experimental cocaine abuse. I. Dose-dependent activation of lymphocyte subsets

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## SUMMARY

The potential role of substance abuse, especially cocaine and alcohol as co-factor in HIV infection and in the development and expression of AIDS has been suggested, but the possible biological role of substance abuse in the development of AIDS is not known. In order to better understand immune system function in chronic cocaine abuse, we have assessed primary B cell responses to helper T-cell independent (TI) and dependent (TD) antigens in inbred Fisher male rats injected with 1·25–5 mg cocaine/kg body weight for 10 days. The ability of cocaine-exposed animals to mount primary *in vivo* splenic plaque-forming cell (PFC) and serum antibody responses to the TI antigen, pneumococcal polysaccharide type III (SIII), was elevated several-fold when compared with controls. The degree of elevation of humoral antibody responses seemed to be directly related to the dose of cocaine. Primary *in vivo* B cell responses to the TD antigen, sheep red blood cells (SRBC), was elevated at lower concentrations of cocaine (1·25–2·5 mg/gk) and was found to be significantly suppressed after chronic exposure to the higher concentration (5·0 mg/kg). The elevated primary splenic immunostimulation to TI (SIII) may be attributed to a combination of loss of T suppressor cell control and direct B cell stimulation. Elevated immune responses to SRBC at lower concentrations were attributed to stimulation of T helper cells as well as loss of T suppressor cells. Immunosuppression to SRBC observed in response to 5·0 mg/kg of cocaine was most probably due to loss of T helper cell subset functions. These findings were further tested by *in vitro* methods, where splenic lymphocytes from cocaine-exposed animals were examined for their ability to respond to concanavalin A (Con A) in terms of the induction of antigen non-specific suppressor T cells. The addition of Con A-stimulated splenic lymphocytes from cocaine-treated animals did not inhibit the primary antibody responses of SRBC as compared with saline-treated controls, indicating that suppressor T cells malfunction after cocaine exposure. Lymphocyte subpopulation analysis using fluorescein-labelled monoclonal antibodies showed a significant increase in the B cell populations at doses of 1·25–5 mg/kg. A reciprocal change in T cell populations also took place. No significant numerical change in macrophage (NSE+) and T cell subset, T helper and T suppressor was noticed, suggesting that cocaine probably directly effects mature T cell subset functions but does not affect their differentiation. *In vitro* exposure of isolated lymphocytes to various doses of cocaine did not result in any significant immunomodulation, indicating that not cocaine but its by-products or a metabolite of cocaine or the stimulation by cocaine of the release of some host factor(s) is responsible for the observed immunological alterations.

**Keywords** cocaine T cell activation B cell proliferation HIV reactivation immunomodulation

## INTRODUCTION

It is estimated that every day approximately 5000 people in the United States try cocaine for the first time. There are about 5 million regular users of cocaine in the U.S. (Quinn *et al.*, 1988), and it is also estimated that about 30 million Americans have tried cocaine at least once (Levy *et al.*, 1986; Des Jarlais *et al.*, 1987). Despite worldwide abuse of cocaine, there has been little research on the effect of the drug on the immune system. The need for such studies has become more important because of the high prevalence of AIDS among cocaine abusers (Sterk, 1988; Chaisson *et al.*, 1989).

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We exposed rats to various concentrations of cocaine (1·25 to 5·0 mg/kg body weight) and found that they respond to helper T cell independent (TI) antigens with plaque-forming cell (PFC) and serum antibody responses that significantly exceed those of controls. Since this elevated immune responsiveness occurs even in an environment in which helper T-cell dependent (TD) antibody responses are significantly depressed (at 5 mg/kg), we suggest an early loss of T suppressor cell function with the administration of lower doses of cocaine. An additional stimulation of B lymphocytes by a higher dose of cocaine may be responsible for the observed immunostimulation.

## MATERIALS AND METHODS

*Animals*

Inbred male Fisher rats (Charles River Breeding Laboratories, Wilmington, MA), initially weighing 150–160 g, were injected with cocaine, 1·25 5–5 mg/kg per day for 10 days according to the method described by Ramos-Aliga & Werner (1982). Six rats per experimental variable were used.

*Antigens*

Purified pneumococcal polysaccharide type III (SIII) was a generous gift from Dr Phillip J. Baker, Division of Microbial Immunity, NIH, Bethesda, MD. The immunological properties of SIII from this source have been described previously (Mansfield & Bagasra, 1978; Bagasra *et al.*, 1987). Sheep red blood cells (SRBC) obtained from a single animal source were purchased from GIBCO (Grand Island, NY) stored in Alsever's solution, and washed three times in sterile saline before use.

*Immunization*

Antigens were diluted in sterile saline and were injected i.p. in 0·2-ml volumes at desired intervals (see Fig. 1). A predetermined optimal dose of 0·5 μg SIII was injected per rat. An optimal dose of $3 \times 10^8$ SRBC was injected by a similar route (Bagasra *et al.*, 1987).

*PFC cell assays*

These assays were performed 5 days post-immunization. Single-cell suspensions were prepared by gently cutting the spleens from various animals into small fragments in ice-chilled RPMI-1640 plus 10% fetal calf serum (FCS) and 50 μg/ml gentamycin. Subsequently, the spleen fragments were gently passed through sterile 60-mm stainless steel mesh screens. Cell debris was removed by incubating the cell suspensions at room temperature and allowing it to settle for 5 min. Cells were washed twice by centrifugation at 400 *g* for 10 min and resuspended in serum-free medium.

*PFC for SIII and SRBC*

For the purpose of demonstrating PFC specific for SIII, target SRBC were coated with SIII, according to the method described in detail previously (Bagasra *et al.*, 1987). PFC against SIII and SRBC were developed as described (Bagasra *et al.*, 1987).

*Microtitre haemagglutination*

Microtitre haemagglutination titres against SRBC and SIII-specific serum antibody responses were measured by passive haemagglutination using heat-inactivated (56°C for 30 min) sera. The method has been described previously (Bagasra *et al.*, 1987).

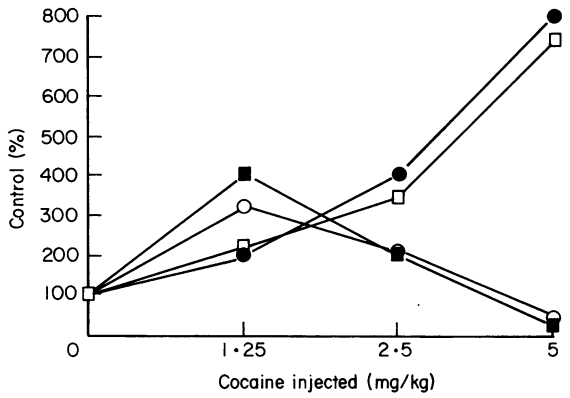


**Fig. 1.** Primary splenic PFC and serum antibody response in rats injected with various doses of cocaine to T-helper-dependent/SRBC (■/○) and T-helper-dependent SIII (□/●). Cocaine-injected and saline-injected (control) rats were immunized with either SRBD ($3 \times 10^8$/animal) or SIII (0·5 μg/ml), and responses were measured 5 days post immunization. Mean IgM counts are shown as PFC/$10^6$. Mean responses are the direct results from six animals per experimental variable; experiments were repeated three times; results of one representative experiment are shown.

*Generation of non-specific suppressor T (Ts) cells*

Lymphocytes from normal or cocaine-exposed animals ($1 \times 10^7$ splenic lymphocytes/well) were cultured in medium containing 2 μg/ml of concanavalin A (Con A). After 48 h these Con A-treated cells were harvested, washed three times in sterile serum-free medium, and passively transferred with an equal number of normal spleen cultures plus 0·05% (v/v) SRBC. These cultures were then addressed for primary antibody response to SRBC 6 days later. Some of the Con A-treated cells were enriched for T cells by passing them over the nylon wool columns. Control cultures received Con A-stimulated normal lymphoid cells that were pretreated with anti-T cell sera plus complement before transfer (Bagasra, Kushner & Hashemi, 1985), to demonstrate that the transferred suppressor cells is a T suppressor cell.

*Subpopulation cell enumeration*

Cell suspensions from spleens were prepared from non-immunized, cocaine- or saline-exposed (control) rats in order to enumerate various cell populations. Enumerations were performed as described previously (Bagasra *et al.*, 1987). Monoclonal antibodies (all purchased from Accurate Chemical Co., Westbury, NY) used for enumeration were as follows: for *pan*-T cell, MRC OX-7 (MAS-027b, an anti-rat Thy 1.1); for T-helper cells, W3 25 (MAS 113B); for T-suppressor cells, MRC OX-8 (MAS 041b); and for B cells, MRC OX-4 (MAS 029b, anti-Ia). Macrophages present in the spleen were identified by counting the number of non-specific esterase-positive (NSE+) cells.

*Statistical analysis*

Tests for statistical differences in the splenic cell subpopulations, PFC responses and ConA-stimulated generation of T suppression cells in the experimental *versus* control animals were

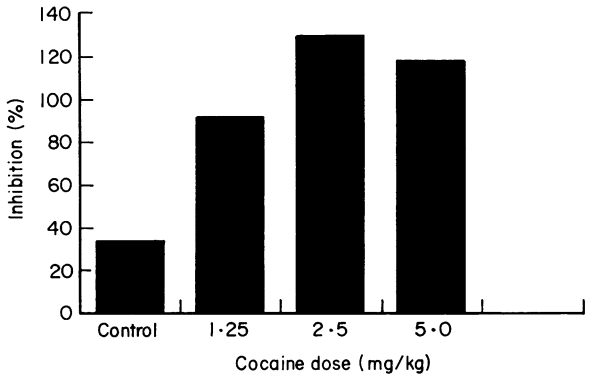


**Fig. 2.** Generation of suppressor T cells. Splenocytes from control and cocaine-injected rats were cultured for 48 h with 2 μg/ml of ConA, washed and added to equal numbers of normal splenocytes + SRBC, and addressed for their ability to inhibit the primary PFC responses *in vitro*. Splenocytes from six animals were used for each experimental variable.

performed by using the Student's *t*-test derived from the responses of a minimum of six animals per experimental variable.

## RESULTS

*Immune responses to helper TI antigens*

Cocaine-injected rats were immunized with SIII at the end of 10-day cocaine exposure, and the magnitude of the subsequent primary antibody responses was measured. Preliminary experiments revealed that the peak day for a primary *in vivo* splenic PFC response to an optimal dose of SIII was day 5 post-immunization for both cocaine-exposed rats and their controls (not shown). As shown in Fig. 1, elevated primary PFC responses were observed 5 days post-immunization at all concentrations of cocaine tested (225–757% of controls, $P < 0{\cdot}001$). SIII-specific serum antibody responses monitored by passive haemagglutination test with SIII-coated SRBC paralleled the PFC responses. The degree of elevated antibody responses was directly related to the increasing dose of cocaine. The peak day of PFC response to SIII was the same for cocaine-exposed and control rats.

*Immune responses to helper TD antigens*

Primary B cell responses to this antigen were assessed in cocaine-exposed animals. The peak day of optimal SRBC-specific PFC was day 5 after primary immunization of experimental animals or their controls (not shown). As shown in Fig. 1, SRBC-specific serum antibody levels monitored by haemagglutination tests paralleled the PFC responses. Elevated humoral responses were observed at lower cocaine concentrations: 322% at the 1·25 mg/kg dose and 213% at the 2·5 mg/kg dose. A 49% depression was noticed at the 5·0 mg/kg dose of cocaine.

*Generation of antigen non-specific suppressor T-cell activity*

Although B cell responses to SIII are not dependent upon helper T cells for induction, the magnitude of the developing response is regulated by suppressor T cell subsets (Baker *et al.*, 1973). We asked whether the enhanced responses to SIII and SRBC at lower doses of cocaine observed in cocaine-exposed animals could be attributed to an inability of suppressor T cells to regulate appropriately the antigen specific response; we therefore attempted to generate non-specific suppressor T cells by low-dose Con A (2 μg/ml). Therefore, splenic lymphocytes from controls and cocaine-exposed animals were examined for their ability to respond to Con A in terms of the induction of antigen non-specific suppressor T cells. The mixing of Con A-stimulated splenic lymphocytes from cocaine-exposed rats was unable to inhibit the primary antibody responses of SRBC as compared with control (Fig. 2). These experiments strongly indicate that non-specific suppressor T cells cannot optimally be stimulated after *in vivo* exposure to cocaine.

**Table 1.** Alteration in splenic cell populations after exposure to cocaine

| Cocaine injected (mg/kg body weight) | Lymphocyte subpopulations (%) | | | T-cell subsets | | |
|---|---|---|---|---|---|---|
| | B cells | Thy 1.1 | NSE+ cells | Th | Ts | Null cells |
| 0 | 70±4 | 29±4 | 3·8±1·9 | 41±6 | 37±4 | 21±5 |
| 1·25 | 85±5* | 14±5* | 4·0±1·5 | 42±2 | 43±5 | 14±4 |
| 2·5 | 84±4* | 16±5* | 3·0±1·0 | 41±8 | 39±5 | 20±6 |
| 5·0 | 79±7† | 20±7† | 2·8±2·0 | 42±5 | 40±6 | 18±6 |

Single cell suspension were prepared from the spleens of cocaine-injected rats; six animals per experimental variables were used. Spleens from six saline injected-rats were used as controls.

B cells were enumerated using MoAb MRC OX4 (MAS 02g, anti Ia); T cells by *pan*-T cell MoAb MRC OX7 (MAS 027); and NSE† cells as described by Bagasra *et al.* (1987).

* $P < 0{\cdot}001$.

† $P < 0{\cdot}01$

*Modulations in splenic cellular subpopulations after cocaine injection*

As shown in Table 1, analysis of splenic cellular subpopulations revealed a significant decrease in the *pan*-T cell subset population after injection of various doses of cocaine. A reciprocal increased in B population as measured by anti-Ia antibody was observed. No change in macrophage cell population (NSE+) or T cell subsets were observed at any of the concentrations of cocaine administered.

In vitro *effect of cocaine*

Exposure of lymphocytes from both rats and humans to a wide range of concentrations of cocaine (10 mg/ml to 1 μg/ml) did not demonstrate a stimulatory effect on B or T lymphocyte subsets (data not shown), as measured by $^3$H-thymidine incorporation. These experiments were repeated six times and the results were the same.

## DISCUSSION

Prevalence of HIV seropositivity among substance abusers is disturbingly high. In a recent report by Sterk (1988), HIV seropositivity among i.v. cocaine abusers was found to be 46%, whereas of those who abuse cocaine and crack by non-i.v. route, 84% were seropositive. Similarly, Chaisson *et al.* (1989) have reported that i.v. cocaine use significantly increased the risk of HIV infection, with a seroprevalence of 35% in daily cocaine

users. It is a common belief among AIDS epidemiologists and other AIDS scientists that the high prevalence of HIV infection in drug abusers is due to a 'dis-inhibiting effect' of abused substance, resulting in increased willingness to participate in 'high risk' behaviors (Stall, 1986).

Immunosuppressive effects of alcohol, the most common substance of abuse, have been documented since the time of Robert Koch who reported that alcoholics were the most common victims of cholera (Koch, 1884). Since then hundreds of carefully conducted studies in humans and in animals have documented various adverse effects of alcohol abuse (reviewed in Bagasra, Fitzharris & Bugasra, 1988). Despite a significant increase in cocaine abuse in recent years, few detailed studies regarding the effect of cocaine on the immune system have been conducted.

In the present study we assessed the ability of rats chronically exposed to cocaine to respond to immunization with helper TI and TD antigens. Our observations show for the first time that *in vivo* exposure to cocaine had a profund immunomodulating effect in the animal host, and that a clear dichotomy exists with respect to the ability of animals to mount a primary *in vivo* splenic PFC response to these two types of antigens. Furthermore, activation and suppression of various lymphocyte subpopulations depended on the dose of cocaine to which the animals had been exposed.

The primary PFC responses to SIII in chronically cocaine-exposed rats were elevated at all concentrations tested, relative to the primary response of control animals. The induction of a B cell response to antigens such as SIII is not dependent upon helper T cells or macrophages (Baker *et al.*, 1973, 1974), and thus the primary splenic PFC response of animals to these antigens reflects intrinsic B cell functions (Bagasra, Kushner & Hashemi, 1985; Bagasra *et al.*, 1985, 1987). The B cell responses obtained in the present study suggest that intrinsic B cell function remained intact up to the 5 mg/kg concentrations of cocaine. It was interesting to note that the degree of immuno-enhancement with respect to SIII increased with increasing concentrations of cocaine (Fig. 1) up to 5 mg/kg, which produced the highest degree of elevation in PFC response.

Although B cell responses to SIII are not dependent upon helper T cells for induction, the magnitude of the developing response is normally regulated by T suppressor and amplifier T cells (Baker *et al.*, 1973, 1974). Therefore, elimination of T suppressor cells or a functional defect in this subset will result in greatly enhanced splenic PFC responses to SIII when compared with T cells from intact animals (Bagasra, Kushner & Hashemi, 1985; Bagasra *et al.*, 1985, 1987). Since elevated responses to both TI and TD antigens are noted at lower doses of cocaine, we asked whether an enhanced response to SIII and SRBC observed during chronic cocaine exposure at these lower doses could be attributed to an inability of suppressor T cells to regulate appropriately the SIII- or SRBC-specific response. It has been shown that exposure of lymphocytes to generate non-specific T suppressor cells at mitogenic concentration of ConA (2 μg/ml) could be used to examine the functional capacity of T suppressor cells (Baker *et al.*, 1974; Bagasra, Kushner & Hashemi, 1985). T cells generated by this method inhibit the generation of the PFC specific for SRBC (Baker *et al.*, 1974). The immuno-enhancement to SIII or SRBC that was observed in response to the lower doses of cocaine may be due to a defect in T suppressor cell function or direct B cell activation by cocaine or its by-products. These findings suggest that intrinsic B cell function, as measured by the primary splenic PFC responses to SIII, is intact. The elevated responses that we observed at the lower concentrations of cocaine were due either to a loss of T suppressor cell functions, T suppressor cell depletion, or both. As was noted in Fig. 2, cocaine-exposed animals were unable to generate adequate degrees of T suppressor cells by ConA-stimulation, supporting the hypothesis that T suppressor function was indeed defective even at lower concentrations of experimental cocaine exposure. At the highest concentration of cocaine (5 mg/kg), defective helper T cell function was also noted (Fig. 1).

The findings of the present study are in agreement with previously published reports on the effect of cocaine on the immune system (Faith, 1983; Van Dyke *et al.*, 1986; Havas *et al.*, 1987). Havas *et al.* found that antibody responses to TI antigens such as SIII and to TD antigens such as DNP resulted in a small rise in antibody levels in male mice. However, the immuno-enhancement they observed was not as high as the one we observed. The reason for such a discrepancy seems to be related to differences in the protocols used for the administration of cocaine. Havas *et al.* injected mice three times daily for 4 days following SIII injection. In the present study we exposed our animals to cocaine for 10 days and immunized them after chronic cocaine exposure. Therefore, exposure to cocaine post-immunization may have different immunological consequences than exposure prior to immunization. Van Dyke *et al.* (1986) have reported a four-fold increase in natural killer cell activity after i.v. administration of cocaine at 0·6 mg/kg. Theoretically, the lessening of suppressor T cell control over the immunoregulatory T cell network could produce such a result (Baker *et al.*, 1973, 1974). In mice, Faith (1983) reported an increase in cell-mediated immunity after cocaine exposure and Fuchs *et al.* (1987) have shown an activation of T cell activity in human substance abusers. Again, a defect at the T suppressor level may indirectly allow non-specific activation of other T-cell subsets.

Primary *in vivo* splenic PFC responses to SRBC were different from the responses observed to SIII antigen. At lower concentrations of cocaine (1·25 and 2·5 mg/kg), elevated PFC responses to these antigens were noticed, but at 5 mg/kg the immune responsiveness of the cocaine-exposed rats was significantly below that of controls. This inability of B cells to respond to helper TD antigen was not due to a defect at the level of the B cell, but rather to a functional defect at the level of accessory cells (since B cell functions were intact, as shown by their ability to respond to SIII). Exposure of cells to cocaine *in vitro* did not produce results similar to those obtained *in vivo*.

We were unable to duplicate our *in vivo* observations (immuno-enhancement or immunostimulation); this indicates that either a metabolite of cocaine, or an immunomodulating factor(s) released in response to cocaine, may have been responsible for the observed effect.

We have not addressed the question of how cocaine could be responsible during its abuse for interfering with regulatory T cell functions or B cell stimulation. It is possible that some of the events observed as a result of administration of cocaine may be the indirect effect of cocaine on the neocortex and the neuroendocrine system (more specifically, the regulation of the secretion of β-endorphin (Forman & Estilow, 1988), or dopamine, or both. The various possible underlying mechanisms responsible for the observed abnormalities in the immune

system during chronic cocaine exposure are currently under investigation in our laboratory.

This present investigation was undertaken to demonstrate in detail the immune system dysfunction that may result from the chronic use of cocaine. Our studies strongly suggest that cocaine abuse results in a sequential loss as well as stimulation of various lymphocyte subpopulations of the immune system. B lymphocytes seem to be most sensitive to stimulatory effects of cocaine or its resultant factors. Whereas suppressor T cells probably are most susceptible to cocaine (or its by-products) followed by amplifier helper T cells that are stimulated by low concentrations but suppressed at higher concentrations of cocaine.

Our results may have a direct implication with regard to the mitogenic and stimulatory activities of cocaine (or its by-products) on the T helper (CD4 equivalent) and B cells. Although we have not yet explored the activation of CD4+ cells by cocaine, epidemiologic and human studies strongly suggest that prevalence of HIV-seropositivity in cocaine abusers (Sterk, 1988; Chaisson *et al.*, 1989) as well as development of AIDS in HIV-seropositive cocaine abusers is signficantly high (Levy *et al.*, 1986; DesJarlais *et al.*, 1987; Friedland & Klien, 1987; Moss 1987; Quinn *et al.*, 1987); this would be suggestive from our experimental data: that they predict that the activation of CD4+ cells (due to CD8+ dysfunction) which subsequently enhance the termination of HIV-proviral stage as well as shorten the latency period temporarily increased the CD4+ relative population. This could be understood in context with the life cycle of HIV. For example, after HIV-RNA enters CD4+ cells via CD4 receptors, it transcribed into DNA. The proviral DNA is integrated into the host genone. After integration of the provirus, HIV may enter a latent phase until the infected cells are activated. Immune activation of the infected T-cell can terminate the viral latency (reviewed in Bagasra, Fitzharris & Bagasra, 1988). Therefore, stimulation of the infected cells with various mitogens, drugs, or heterologous viruses can break down HIV-1 latency (Sapolsky *et al.*, 1987; Bagasra 1988; Patarca *et al.*, 1988). Fuchs *et al.* (1987) have reported activation of cellular immunity in substance-abusing patients and in HIV-seropositive individuals.

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